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16. ABSTRACT

A technique has been developed to separate a mixture of the twelve isomeric phenols of benzo(a)pyrene by using High-Performance Liquid Chromatography with fluorescence detection and various sequences of methanol/water gradients. Detection by fluorescence has increased the sensitivity of the method when compared with UV detection. The effects of flow rate, temperature, and various types of gradients on the separation of the isomeric phenols are discussed. It has been found that under the conditions normally employed to separate these phenols from other metabolites of benzo(a)pyrene, several of the phenols are not separated possibly leading to incorrect identification as well as quantitation. Diols and diones of benzo(a)pyrene are also separated by this procedure

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Journal Article

The Separation of the Isomeric Phenols of Benzo(*a*)Pyrene by High-Performance Liquid Chromatography

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A method has been developed to separate the 12 isomeric phenols of benzo(*a*)pyrene by using high-performance liquid chromatography with fluorescence detection and various sequences of methanol/water gradients. The elution of the 12 isomers by different concentrations of methanol/water was studied showing that many isomers have almost identical retention times. The effects of flow rate, temperature, and various types of gradients on the separation of the isomeric phenols, as well as the difficulty to separate some of the isomers, are discussed. It has been found that under the conditions normally employed to separate these phenols from other metabolites of benzo(*a*)pyrene, several of the phenols are not separated possibly leading to incorrect identification as well as quantitation. Detection of the phenols, as well as the parent benzo(*a*)pyrene, by fluorescence has increased the sensitivity of the method about 200 times when using the most sensitive setting as compared to uv detection. All the available pure diols and diones of benzo(*a*)pyrene are also separated by this procedure.

Polynuclear aromatic hydrocarbons (PAH)[3] are environmental pollutants which contaminate air, soils, oceans, rivers, and associated fauna. They are products of incomplete combustion of fossil fuels, major exhaust components of transportation, refuse, burning and industrial energy sources, and some of them have been associated with cancer in man (1). Benzo(*a*)pyrene (BP) is considered a model compound for the PAHs since its metabolism has been widely investigated (2–10). BP and the other PAHs are generally metabolized by microsomal mixed-function oxidases (MFO), which are predominantly found in the liver of many animal species including fish (11–17).

Most of the presently used high-performance liquid chromatography (hplc) methods for separation and identification of BP metabolites are based on the very fine original method of Selkirk *et al.* (8), but will not resolve the isomeric phenols (15,18,19). Confirmation depends principally on the traditional method of cochromatographing certain known standards using linear gradients of water/methanol or water/acetonitrile, in spite of the fact that under these conditions some phenols have identical retention times (8,15,18,20,22). Selkirk *et al.* (23) refined their original work with a method that made use of an elaborate column setup together with recycling the isomers, and found that the 1- and 7-OH had previously cochromatographed with the 3-OH. However, the procedure did not show the position of 2-OH and 11-OH. Recently, Stegeman (15) tried an alkaline gradient system to improve the separation of BP phenols using a highly alkaline eluant (pH > 11.5) consisting of a

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[3] Abbreviations used: PAH, polynuclear aromatic hydrocarbons; BP, Benzo(*a*)pyrene; MFO, mixed-function oxidases.

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series of alkylamines in a linear gradient mode. This procedure did not, however, separate other BP metabolites such as diols and quinones, and the high pH may be damaging to the column (24).

A convenient and sensitive reverse-phase hplc method for separation of BP phenols, dihydrodiols, and quinones has been developed which allows for the precise identification of all phenols of BP.

EXPERIMENTAL

Apparatus. A Perkin–Elmer 3B liquid chromatograph was used during the regular solvent program separation; a Spectra-physics SP8000B liquid chromatograph was used for the combined flow and solvent programming and a Perkin–Elmer HC-ODS SIL-X reverse-phase column (0.26 × 25 cm) was used for separation of the isomers; other columns tried were: Whatman partisil ODS, DuPont Zorbax C_8, and Zorbax ODS. A Schoeffel FS 970 fluorometer (excitation 263-nm and emission 370-nm filter) and a Schoeffel FS 770 uv (monitored at 254 nm) detectors fitted with 8.0 μl flow-thru cuvettes were used for detection and characterization of BP derivatives.

Reagents and standards. BP, its phenols, dihydrodiols, and diones, obtained through NCI Chemical Repository Laboratory at IIT Research Institute, Chicago, Illinois, were tested for purity by hplc using the gradient system proposed in this article. Methanol, acetone, and ethyl acetate were LC grade from Burdick and Jackson Laboratories. The water used was deionized, distilled over potassium permanganate, redistilled twice, and stored in dark containers.

Preparation of standards. All work was carried out under red light. The concentrated stock standard solutions were prepared by direct weighing, dissolved in absolute methanol, and stored under nitrogen at −80°C. The working standard solutions were prepared by diluting certain volumes of stock solution with methanol and were stored under nitrogen at −80°C. The concentrations of the working standards generally ranged from 0.100 to 0.600 ng/μl with an injected volume of 10.0–20.0 μl.

Mixtures of the 12 isomeric phenols were prepared by taking an aliquot of each stock solution (10.0–20.0 μl) and bringing the total volume to 10.0 ml with methanol. The gradient system was composed of 90/10 methanol/water (Solvent A) and 30/70 methanol/water (Solvent B), and was run at constant flow, except for the separation of 12-OH and 11-OH isomers.

RESULTS AND DISCUSSIONS

Use of a reverse-phase ODS C-18 column in conjunction with a new gradient system has enabled us to resolve BP derivatives, especially phenols, more efficiently than previously reported (8,11,15,16,18,21–23). This method has the advantage of separating biologically produced BP metabolites, diols, diones, phenols, and unmetabolized BP on one chromatogram.

During the development of the method of separation, the solvent gradient was found to be the most important aspect to consider for increasing the resolution of the isomeric phenols. Preliminary studies using linear gradients with various proportions of water in methanol resulted in poor resolution; only five peaks were obtained for the 12 isomers, therefore, each peak may have contained 2 or more isomers. The 5-OH may have eluted along with 11-OH and 12-OH, or possibly with the 4-OH. The 6-OH may have appeared in the peak area of the 9-OH, while the 8-OH and 10-OH were unseparable from each other and from either the 2-OH or the 9-OH. The 7-OH may have appeared in the peak area of the 2-OH or the 1-OH. On the other hand, the following isomers are separated in this order: 4-OH, 9-OH, 2-OH, 1-OH, and 3-OH. Different types of linear gradients using methanol and water resulted in little improvement in resolution. Con-

cave or convex gradients produced six peaks.

Initially, 100% weak Solvent B (30/70 methanol/water) was used as eluting solvent. The strong Solvent A (90/10 methanol/water) was used to increase the amount of methanol in a stepwise pattern. This technique has been found to be very important in achieving the slow separation of the unresolved peaks. The quality of separation and the number of separated peaks will depend on both the length and the solvent composition of each segment of the gradient. Figure 1 shows the separation of a mixture of phenols obtained under the specified conditions. The mixture contained 10 of the 12 BP phenols, but did not include the 11-OH and 8-OH because of coelution with the 12-OH and 10-OH, respectively. When the flow rate and gradient composition were modified as specified in the legend of Fig. 2, the separation pattern of 11 isomers was as shown in this chromatogram. The slower rate is thought to provide a more efficient separation. It must, however, be accompanied by moderate changes in solvent composition since a sudden change in gradient composition from 0 to 45% Solvent A will cause the 4-OH and 5-OH peaks to merge. We conclude that a moderate change in solvent composition earlier in the gradient program is very critical for separation of the 4-OH and 5-OH isomers as shown in Fig. 3.

Further improvement was obtained by using the microprocessor controlled gradient system of Perkin–Elmer 3B which has five programmable time segments, each of which allows the use of 1 of 10 different gradient types (N = 0 to 5). Each gradient curve is presented by N in the equation

$$P(t) = P(i) + [P(f) - P(i)]\left(\frac{t}{T}\right)^{N},$$

where

$P(t)$ = A/A(A + B)% which is solvent A%
$P(i)$ is the initial A/(A + B)%

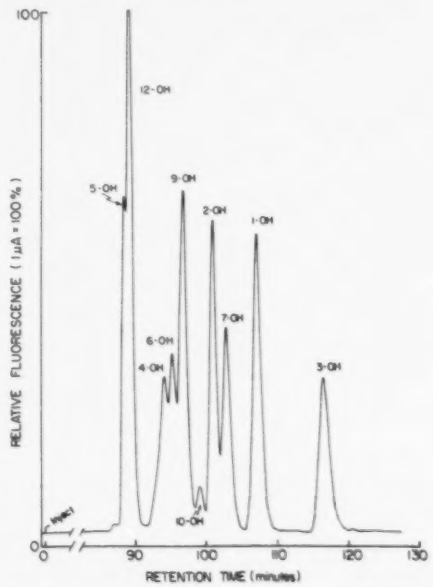

FIG. 1. Separation profile of a mixture of 10 phenols of BP obtained from a Perkin–Elmer ODS SIL-X column; flow rate 0.6 ml/min; temperature 35°C. The gradient system consisted of Solvent A (90/10, methanol/water) and Solvent B (30/70 methanol/water) and was changed as follows: Solvent B was used to equilibrate the column for 20 min before injection, followed by stepwise increases of Solvent A: 0% for 15 min, 10% for 15 min, 25% for 23 min, 35% for 10 min, 45% for 6 min, 60% for 5 min, and 70% for 45 min. Identity of peaks was based on results obtained by injection of individual standards, pairs of standards, and doubling the concentration of one standard in the mixture each time.

$P(f)$ is the final A/(A + B)%
t is the elapsed time (minutes)
T is the length of each time segment (minutes)
N is the gradient curvature

A mixture containing eight phenols was separated in the following order, 5-OH, 4-OH, 6-OH, 9-OH, 10-OH, 2-OH, 7-OH, and 1-OH (Fig. 3). The 3-OH was found to elute after the 1-OH under all conditions used. Improvement was obtained (Fig. 4) with a gradient that had two concave segments (N = 5) at the beginning followed by the regular stepped pattern of gradient

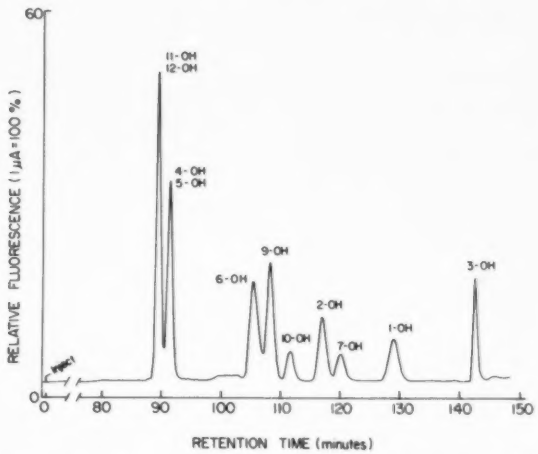

FIG. 2. Separation profile of a mixture of twelve phenols of BP obtained from a Perkin–Elmer ODS SIL-X column; flow rate 0.3 ml/min, temperature 35°C. The gradient system consisted of Solvent A (90/10, methanol/water) and Solvent B (30/70 methanol/water) and was changed as follows: Solvent B was used, at flow rate 0.6 ml/min, to equilibrate the column for 20 min before injection, followed by stepwise increases of Solvent A: 0% for 10 min, 45% for 30 min, 55% for 8 min, 60% for 8 min, 65% for 10 min, 75% for 60 min, and 100% min for 20 min. Identity of peaks was based on results obtained by injection of individual standards, pairs of standards, and doubling the concentration of one standard in the mixture each time.

changes when run at flow rate of 1.0 ml/min. This resulted in the following separation: 4-OH plus 5-OH, 6-OH, 9-OH, 8-OH, 10-OH, 2-OH, 1-OH, and 3-OH. We found (Fig. 5) that by changing the flow rate from 1.0 ml to 0.6 ml/min using the same gradient conditions, as appeared in the legend of Fig. 5, it was possible to separate diols, diones, phenols of BP, as well as to differentiate between the *cis*- and *trans*-isomers of the 7,8-diol on one chromatogram.

We used only two of the diones 1,6- and 3,6-isomers, in the chromatogram shown in Fig. 5 because of the fact that the available 6,12-dione was impure as were some of the diols. However, recently we observed that the 6,12-dione would elute directly after the 1,6-isomer.

Flow rates ranging from 0.3 to 2.0 ml/min were tested to study their effects on quality of separation. A flow rate of 0.6 ml/min when used along with a proper solvent composition (Fig. 1) resulted in a good separation of 10 phenols. However, a slower flow rate of 0.3 ml/min and gradient system as used to obtain the chromatogram shown in Fig. 2 did not improve the separation of the first four isomers, but it improved the separation of the other phenols to a baseline resolution. To improve the separation of these four isomers, any drastic changes must be prevented early in the gradient program. This should be done in a way that does not affect the quality of the separation of the other isomers. Therefore, the concave gradient curve of $N = 5$ was entered as a gradient parameter for the first two time segments. In order to obtain a favorable separation at 1.0 ml/min, the gradient conditions had to be changed as shown in Fig. 3. Flow rates higher than 1.0 ml/min caused very high column back pressure at the beginning of the separation, due to the high viscosity of Solvent B, which in turn affects the normal separation pattern.

Since separation of the 12-OH and 11-OH isomers was next to impossible under the above-mentioned conditions, a somewhat different approach was used as described later.

The behavior of each isomer in different methanol/water proportions was also investigated. Table 1 shows retention times of the isomers injected singly and eluted isocratically with 60, 70, 80, 90, and 100% Solvent A in Solvent B. The retention times listed in this table could explain why some isomers

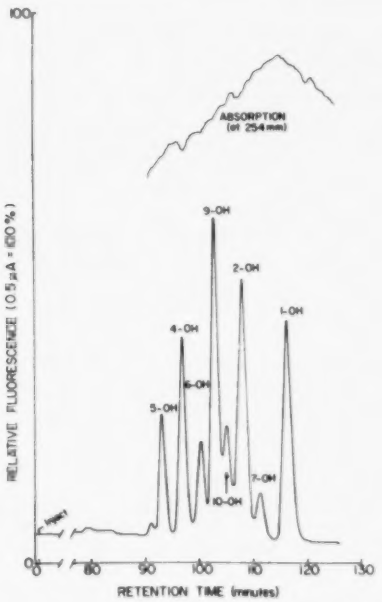

FIG. 3. Separation profile of a mixture of eight phenols of BP obtained from a Perkin–Elmer ODS SIL-X column; flow rate 1.0 ml/min; temperature 35°C. The gradient system consisted of Solvent A (90/10 methanol/water) and Solvent B (30/70 methanol/water) and was changed as follows: Solvent B was used, at flow rate 0.6 ml/min, to equilibrate the column for 20 min before injection, followed by stepwise increases in Solvent A: 1% for 10 min, 10% for 10 min, 20% for 20 min, 30% for 30 min, 30–45% in 15 min of convex gradient (N = 0.4), and 45–60% in 40 min of convex gradient (N = 0.4). Identity of peaks was based on results obtained by injection of individual standards, pairs of standards, and doubling the concentration of one standard in the mixture each time. The uv detector sensitivity was (A = 0.02).

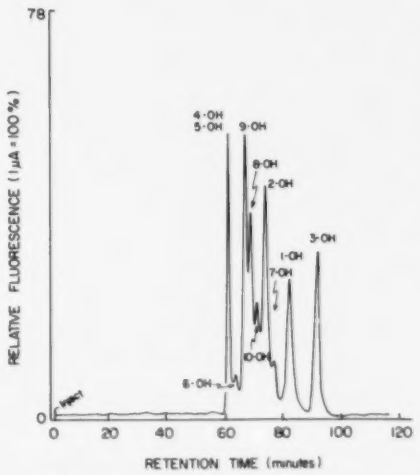

FIG. 4. Separation profile of a mixture of ten phenols of BP obtained from a Perkin–Elmer ODS SIL-X column; flow rate 1.0 ml/min, temperature 30°C. The gradient system consisted of Solvent A (90/10 methanol/water) and Solvent B (30/70 methanol/water) and was changed as follows: Solvent B was used, at flow rate 0.6 ml/min, to equilibrate the column for 20 min before injection, followed by increases of solvent A: 1–15% in 10 min of concave gradient (N = 5), 15–25% in 15 min of concave gradient (N = 5), 30% for 15 min, 45% for 15 min, and 60% for 100 min. Identity of peaks was based on results obtained by injection of individual standards, pairs of standards and doubling the concentration of one standard in the mixture each time.

are ready to separate while others show difficulty in separation. For example, the retention time values of the 12-OH and 11-OH at the different solvent compositions used seem to be identical except for the 1-min difference in their retention times at 70% A, which is not sufficient for separation. Some other isomers listed in the table (6-OH to 3-OH) have shown significant changes in retention time versus changes in solvent composition. This is in agreement with the fact that the less methanol in the solvent composition, the longer is the relative retention of the isomers. Hence, the established gradient system allows the column to retain these isomers for enough time to affect separation. It has been found that during the gradient program, the sol-

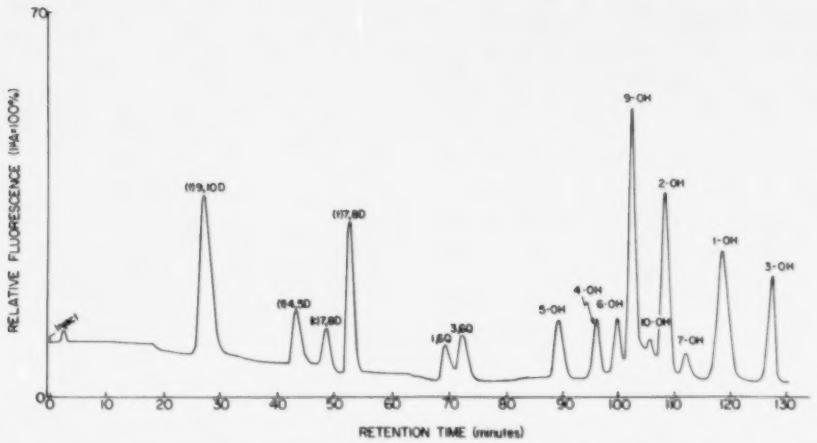

FIG. 5. Separation profile of a mixture of diols, diones, and nine phenols of BP obtained from a Perkin–Elmer ODS SIL-X column; flow rate 0.6 ml/min; temperature 30°C. The gradient system consisted of Solvent A (90/10 methanol/water) and Solvent B (30/70 methanol/water) and was changed as follows: Solvent B was used to equilibrate the column for 20 min before injection, followed by increases in Solvent A: 1–15% in 10 min of concave gradient (N = 5), 15–25% in 15 min of concave gradient (N = 5), 30% for 15 min, 45% for 15 min, and 60% for 100 min. Identity of peaks was based on results obtained by injection of individual standards, pairs of standards, and doubling the concentration of one standard in the mixture each time. Symbols: (t) = *trans*; (c) = *cis*; D = benzo(*a*)pyrene dihydrodiol; Q = benzo(*a*)pyrene dione.

vent composition should not be changed to more than 75% A before the complete elution of the 9-OH, 8-OH, 10-OH, 2-OH, and 7-OH, otherwise most of these isomers will coelute with each other. This explains the incomplete separation of some phenolic isomers when a linear gradient up to 100% methanol was used. If the solvent composition drops below 60% A, unacceptable wide peaks result.

Consulting Table 1, and in particular the data for the retention times of the 9-OH, 8-OH, 10-OH, 2-OH, and 7-OH isomers, it becomes apparent that any possible resolution would occur only at lower than 80% A. This is especially true for the 8-OH, 10-OH, and 2-OH where 60% A gives only 1-min separation at a total time of around 38 min, but unlike the 11-OH and 12-OH, these isomers can be separated by the programmed gradient method. In order to reasonably separate the 8-OH from the 10-OH the gradient system and the flow rate were modified as shown in Fig. 4. The use of 1.0 ml/min flow rate is very important to permit a comparatively larger volume of the

TABLE 1

RETENTION TIMES OF BP-PHENOLS IN DIFFERENT METHANOL/WATER CONCENTRATIONS ON ODS SIL X COLUMN

BP-phenols[b]	Retention time[a] (min) when run with				
	100% A	90% A	80% A	70% A	60% A[c]
12-OH	2.6	3.5	5.3	12.4	16.0
11-OH	2.9	3.3	5.7	11.4	16.1
5-OH	2.8	3.4	6.6	14.0	18.3
4-OH	2.8	3.6	6.4	12.7	18.5
6-OH	3.1	4.6	9.5	16.9	25.7
9-OH	3.3	5.0	10.4	18.6	29.6
8-OH	3.9	5.3	11.1	20.2	37.4
10-OH	3.3	4.5	10.9	19.3	38.6
2-OH	3.6	5.5	11.5	21.9	39.4
7-OH	3.9	5.5	11.4	21.6	44.7
1-OH	4.2	6.3	13.2	23.2	51.2
3-OH	4.9	8.0	17.0	29.1	64.9

[a] The column was equilibrated with the indicated solvent for 20 min before injection. Flow rate 0.6 ml/min, temperature 35°C.

[b] Listed in order of elution under new gradient system.

[c] Solvent composition: (A) 90/10 methanol/water and (B) 30/70 methanol/water.

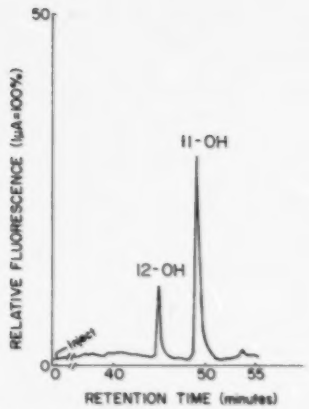

FIG. 6. The separation of 12-OH and 11-OH BP obtained from the SP8000B LC and a Perkin–Elmer ODS SIL-X column, temperature 30°C; the column was equilibrated by Solvent B for 20 min at flow rate 0.6 ml/min. Both flow rate and gradient composition were changed, as injection started, as follows: Solvent A: 0% at flow rate of 0.4 ml/min for 20 min then changed to 30% A at flow rate 0.6 ml/min for 5 min, followed by a final segment of the gradient of 70% A at a flow rate of 0.9 ml/min, and kept under this condition until the end of the run. Identity of the peaks was based on results obtained by injection of individual isomers and doubling the concentration of one standard in the mixture each time.

low-methanol gradient to initially pass through the column for more efficient separation of these two isomers.

Separation of the 12-OH and 11-OH isomers was obtained using both programmed flow and programmed gradient and is illustrated in Fig. 6. The best separation was obtained when the initial part of the gradient (0% A) was delivered at a flow rate of 0.4 ml/min for 20.0 min, then changed to 30% A at 0.6 ml/min for 5.0 min, followed by a final segment of the gradient at 70% A and a flow rate of 0.9 ml/min.

The relative fluorescence of each isomer emerging from the column in differing percentages of water was found to decrease with increasing concentration of water. The 3-OH, 5-OH, 6-OH, 7-OH, and 1-OH showed a decrease in relative fluorescence of 60–85% at 66/34 methanol/water when compared to methanol. Some preliminary work to establish the cause for this behavior (complete data to be published) done in this laboratory shows that the relative fluorescence activity of some phenols is reduced in presence of water. Column binding with increasing water concentrations is another possible cause for the reduced, emergent fluorescence peaks. At present, it appears that both effects occur, but may work in combination only in the case of some of the isomers. To eliminate these effects when quantitative work is desired, the following method, utilizing peak area calculations, is currently used in this laboratory: plotting the relative fluorescence energy against differing concentrations of each standard eluted under exactly the same condition and extrapolating to the true concentrations from this graph. In addition cochromatography is used for both qualitative and quantitative confirmation.

The fluorescence detection of the phenols, diols, and the parent benzo(*a*)pyrene has increased the sensitivity of the method, when compared to uv absorption, about 200 times depending on each isomer. This comparison is based on the use of the highest detector sensitivity-to-noise ratio of the Schoeffel FS 970 fluorometer and the highest detector sensitivity-to-noise ratio of the SF 770 uv detector. Figure 3 shows a representative comparison between uv and fluorescence detection.

The effect of the column temperature on the quality of separation and the stability of thermolabile metabolites was also studied. A column temperature of 30–35°C is convenient for the compounds and would not affect separation pattern. Higher temperature, e.g., 50°C, causes sharper, rapidly eluted peaks, but poor resolution. The 6-OH was unstable even at −20°C and decomposed to give three peaks on hplc, two of which were identified as the 3,6- and 1,6-diones. Consequently, all 6-OH standards were used within hours of their preparations.

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